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Identification and characterization of *Bacillus anthracis* by multiplex PCR analysis of sequences on plasmids pXO1 and pXO2 and chromosomal DNA

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Abstract

Bacillus anthracis can be identified on the basis of the detection of virulence factor genes located on two plasmids, pXO1 and pXO2. Thus isolates lacking both pXO1 and pXO2 are indistinguishable from closely related *B. cereus* group bacteria. We developed a multiplex PCR assay for characterization of *B. anthracis* isolates, and simultaneous confirmation of the species identity independent of plasmid content. The assay amplifies *lef*, *cya*, *pag* (pXO1) and *cap* (pXO2) genes, and a *B. anthracis* specific chromosomal marker, giving an easy-to-read profile. This system unambiguously identified virulent ($pXO1^+/2^+$) and avirulent ($pXO1^+/2^-$, $pXO1^-/2^+$ and $pXO1^-/2^-$) strains of *B. anthracis* and distinguished 'anthrax-like' strains from other *B. cereus* group bacteria.

Keywords: Bacillaceae; *Bacillus anthracis*; *Bacillus cereus*; *Bacillus anthracis* genetics; Anthrax epidemiology; Bacterial identification; Diagnosis; Polymerase chain reaction

1. Introduction

Bacillus anthracis is a human pathogenic bacillus. It can cause anthrax, a serious and often fatal infection in both livestock and humans. Animals become infected after coming into contact with soil-borne spores when grazing. Humans become infected only incidentally when brought into contact with diseased animals or their waste products. Other *Bacillus* species are saprophytic and are found in soil, vegetation, air and water and some species such as *B. cereus* are responsible for benign food poisoning [1].

The current laboratory diagnosis for *B. anthracis* is the microbiological analysis of morphological and physiological characteristics. The main characteristic used to distinguish *B. anthracis* from closely related soil-borne *Bacillus* is the presence of two virulence

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Table 1
Strains tested and genotyping of *B. anthracis* isolates examined

Name	CEB no.	Original ID	Plasmid content	Single PCR results (58°C)				
				lef 3–4	*cya* 25–26	*pag* 67–68	*cap* 57–58	Ba813 R1–R2
Clostridium botulinum A	94-7	CEB	na	–	–	–	–	–
Yersinia pestis	na	31-93	na	–	–[a]	–	–	–
Yersinia enterocolitica	85-15	CEB	na	–	–[a]	–	–	–
Streptococcus pyogenes A	90-6	CEB	na	–	–	–	–	–
Staphylococcus aureus	90-1	CowanIII	na	–	–	–	–	–
Escherichia coli	85-35	CEB	na	–	–	–	–	–
Citrobacter freundii	86-45	CEB	na	–	–	–	–	–
Enterobacter aerogenes	86-46	CEB	na	–	–	–	–	–
Proteus mirabilis	86-51	CEB	na	–	–	–	–	–
Providencia rettgeri	86-52	CEB	na	–	–	–	–	–
Shigella boydii	86-58	CEB	na	–	–	–	–	–
Salmonella arizonae	87-12	CEB	na	–	–	–	–	–
Pseudomonas aeruginosa	90-27	CEB	na	–	–	–	–	–

Name	CEB no.	Original ID	Plasmid content	Multiplex PCR results (58°C)				
				lef 3–4	*cya* 25–26	*pag* 67–68	*cap* 57–58	Ba813 R1–R2
Bacillus mycoides	96-12	ATCC 6462	na	–	–	–	–	–
Bacillus cereus	92-11	ATCC 14579	na	–	–	–	–	–
Bacillus thuringiensis	92-6	ATCC 10792	na	–	–	–	–	–
Bacillus licheniformis	92-4	ATCC 14580	na	–	–	–	–	–
Bacillus stearothermophilus	92-5	ATCC 7953	na	–	–	–	–	–
Bacillus sphaericus	92-7	ATCC 14577	na	–	–	–	–	–
Bacillus alvei	92-8	ATCC 6344	na	–	–	–	–	–
Bacillus megaterium	92-9	ATCC 14581	na	–	–	–	–	–
Bacillus pasteurii	92-10	ATCC 11859	na	–	–	–	–	–
Bacillus pumilus	92-3	ATCC 7051	na	–	–	–	–	–
Bacillus subtilis	92-2	ATCC 6051	na	–	–	–	–	–
Bacillus subtilis	92-12	ATCC 9372	na	–	–	–	–	–
Bacillus anthracis	95-31	Sterne 7700	$pXO1^-/2^-$	–	–	–	–	+
Bacillus anthracis	95-32	Sterne 7702	$pXO1^+/2^-$	+	+	+	–	+
Bacillus anthracis	95-36	ATCC 4229	$pXO1^-/2^+$	–	–	–	+	+
Bacillus anthracis	95-33	ATCC 6602	$pXO1^-/2^+$	–	–	–	+	+
Bacillus anthracis	96-6	Cepanzo	$pXO1^+/2^-$	+	+	+	–	+
Bacillus anthracis	95-34	Davis TE 702	$pXO1^-/2^+$	–	–	–	+	+
Bacillus anthracis	227	This study	$pXO1^-/2^+$	+	+	+	+	+
Bacillus anthracis	957	This study	$pXO1^+/2^-$	+	+	+	–	+
Bacillus anthracis	170	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	300	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	779	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	832	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	663	This study	$pXO1^-/2^+$	+	+	+	+	+
Bacillus anthracis	376	This study	$pXO1^-/2^+$	+	+	+	+	+
Bacillus anthracis	846	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	256	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	582	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	282	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	85	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	576	This study	$pXO1^+/2^+$	+	+	+	+	+
Bacillus anthracis	955	This study	$pXO1^+/2^+$	+	+	+	+	+

Table 1 (continued)

Name	CEB no.	Original ID	Plasmid content	Single PCR results (58°C)				
				lef 3–4	*cya* 25–26	*pag* 67–68	*cap* 57–58	Ba813 R1–R2
Bacillus anthracis	554	This study	pXO1⁻/2⁻	–	–	–	–	+
Bacillus anthracis	657	This study	pXO1⁻/2⁺	+	+	+	+	+
Bacillus anthracis	516	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	346	This study	pXO1⁺/2⁻	+	+	+	+	+
Bacillus anthracis	552	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	470	This study	pXO1⁻/2⁻	+	+	+	+	+
Bacillus anthracis	575	This study	pXO1⁺/2⁻	+	+	+	–	+
Bacillus anthracis	204	This study	pXO1⁻/2⁺	+	+	+	+	+
Bacillus anthracis	893	This study	pXO1⁺/2⁺	+	+	+	+	+
Bacillus anthracis	69	This study	pXO1⁺/2⁻	+	+	+	–	+

na, not applicable.
[a]See text for details.

plasmids pXO1 (185 kb) and pXO2 (95 kb). Virulence factor genes *lef*, *cya*, *pag* (pXO1) [2–4] or *cap* (pXO2) [5] have been used as markers to detect *B. anthracis* in the environment using the polymerase chain reaction (PCR) [6–8].

pXO1 and pXO2-cured strains have arisen spontaneously from fully virulent precursors [9]. These avirulent strains cannot be distinguished from other *B. cereus* group bacteria by plasmid analysis. Other conventional methods including gamma phage sensitivity test or carbohydrate profile have to be used. DNA homology studies [10] have shown that *B. anthracis* is very closely related to *B. cereus*, *B. thuringiensis* and *B. mycoides*. The taxonomy of this group is controversial because of the many similarities between the nucleotide sequences of 16S rRNA and 23S rRNA genes, and 16S to 23S intergenic spacer region in the various isolates [11–13]. Nevertheless, genetic variability among *B. anthracis* and other *B. cereus* group bacteria has been reported [14–17]. As an example, the variable region with repetitive sequences (*vrrA*) diverged only slightly between strains of anthrax but is substantially different in *B. cereus* and *B. mycoides* [16]. More recently, a DNA sequence (Ba813) isolated from *B. anthracis* was described as a specific chromosomal marker for this species [17]. Ba813 is a 277 bp long DNA sequence and is present in a single copy in the chromosome of *B. anthracis*. Nucleotide sequence and deduced amino acid sequence comparisons with previously described sequences revealed no homology [17], thus providing a simple way to differentiate *B. anthracis* from the *B. cereus* group.

The increasingly frequent isolation of avirulent *B. anthracis* from humans and animals [9,18] is creating a need for a rapid identification system. The purpose of this study was to develop a PCR-based identification system for *B. anthracis* with the following characteristics: (i) screening for the presence of pXO1 and pXO2, (ii) identifying all known virulence factors (*lef*, *cya*, *pag* and *cap*) (iii) differentiating *B. anthracis* from other *B. cereus* group bacteria. We report a multiplex PCR assay that should be convenient for most clinical and veterinary laboratories and reliable for plasmid content comparisons between strains.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *B. anthracis* isolates were obtained from various collections in France. Bacilli were cultured as previously described [17]. Other strains were cultured in soy-trypticase broth (BioMérieux).

2.2. DNA preparation

Genomic DNA from *B. anthracis* bacteria was isolated from pure cultures by standard procedures [17].

Control DNAs were extracted from the various species listed in Table 1 [17]. Genomic DNA from *Yersinia pestis* (a strain bearing pFra, pYV and pPst plasmids) was kindly provided by Dr. Elisabeth Carniel (Institut Pasteur, Paris).

2.3. Oligonucleotides

Oligonucleotides R1 and R2 were synthesised previously to amplify by PCR a 152 bp fragment from the sequence Ba813, at 59°C [17]. Two primer pairs were designed from *lef*, *cya*, *pag*, and *cap* sequences [2–5] by computer analysis using the Oligo® primer analysis software (MedProbe, Oslo, Norway). The sequences for these primers were designed to amplify all virulence factor genes and the Ba813 marker using the following guidelines: (i) the annealing temperature should be close to 59°C; (ii) the length of amplification products should be between 1 kbp and 200 bp (Table 2).

2.4. DNA amplification procedure

Each 25 µl reaction mixture contained 200 µM of each dATP, dCTP, dGTP and dTTP, 0.5 µM of primers, 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 13% (w/v) sucrose, 40 mg ml^{-1} cresol red, 1 U of *Taq*-DNA polymerase (Boehringer) and 100 ng of DNA. Amplifications were carried out in a GeneAmp® PCR system 9600 (Perkin Elmer). The initial denaturation at 94°C for 5 min was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The reaction mixtures (10 µl) were directly loaded onto 2% (w/v) agarose (Sea-Kem GTG, FMC Bioproducts) gel and DNA fragments separated at 100 V for 1 h before staining with ethidium bromide.

2.5. Hybridization techniques

PCR products were purified using the Sephaglass band prep kit (Pharmacia) and labelled with [α-^{32}P]dCTP (ICN) and the random priming labelling kit (Boehringer). Dot-blot hybridization was at

Table 2
Sequence and position of the primers used in the polymerase chain reaction

Primers	Loci	Positions[a]	T_m (°C)[b]	T_a (°C)[c]	Length (bp)	Sequence (5′→3′)	Reference
67	*pag*	1925–1944	63	64	747	CAGAATCAAGTTCCCAGGGG	This study
68		2652–2671	63			TCGGATAAGCTGCCACAAGG	
23	*pag*	2006–2027	59	58	151	CTACAGGGGATTTATCTATTCC	This study
24		2135–2156	57			ATTGTTACATGATTATCAGCGG	
65	*cya*	1255–1274	58	60	929	CAGCATGCGTTTTCTTTAGC	This study
66		2164–2183	58			CCCTTAGTTGAATCCGGTTT	
25	*cya*	1459–1478	58	59	546	GGTTTAGTACCAGAACATGC	This study
26		1990–2004	61			CGGCTTCAAGACCCC	
59	*lef*	949– 970	54	60	993	GGATATGAACCCGTACTTGTAA	This study
60		1921–1941	62			TAAATCCGCACCTAGGGTTGC	
3	*lef*	1238–1258	54	54	385	CTTTTGCATATTATATCGAGC	This study
4		1599–1622	57			GAATCACGAATATCAATTTGTAGC	
17	*cap*	1230–1249	54	57	873	GAAATAGTTATTGCGATTGG	This study
20	(B, C, A)	2083–2102	63			GGTGCTACTGCTTCTGTACG	
57	*cap* (C)	1603–1622	58	57	264	ACTCGTTTTTAATCAGCCCG	This study
58		1847–1866	56			GGTAACCCTTGTCTTTGAAT	
R1	Ba813	227– 249	59	58	152	TTAATTCACTTGCAACTGATGGG	[17]
R2		98– 120	60			AACGATAGCTCCTACATTTGGAG	

[a]Positions are given according to published sequences [2–5].
[b]Primer T_m was calculated by the nearest-neighbor method [19].
[c]Optimized annealing temperature.

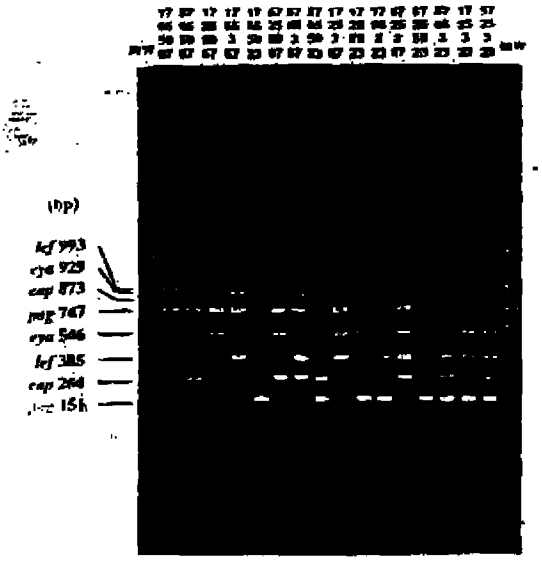

Fig. 1. Simultaneous PCR-based amplification of capsule (*cap*), edema factor (*cya*), lethal factor (*lef*) and protective antigen (*pag*) genes from 100 ng of DNA from the virulent strain of *Bacillus anthracis* CEB779. Primers combinations are indicated on the top: forward primer only shown. MW: *Sau*3A and *Taq*I digested pUC19 DNA size markers: 1444, 955, 735, 584, 476, 341, 258 and 141 bp.

65°C and membranes were washed at low stringency (2SSC, 65°C) before exposure.

3. Results and discussion

3.1. Specificity of oligonucleotide primers

We attempted to amplify the targeted fragments from 100 ng samples of DNA isolated from Sterne 7700, Sterne 7702 and ATCC4229 strains of *B. anthracis*. Fragments of the expected size were successfully amplified from DNA of all appropriate strains of *B. anthracis* (data not shown). The annealing temperature was optimized for each primer pair and the results are given in Table 2. The specificity of oligonucleotide primers was checked by PCR with 100 ng of DNA from other *Bacillus* species and strains belonging to other genera (Table 1). No amplified products were generated from these control DNAs with one exception: small amounts of products of incorrect size were amplified from *Y. pestis* (a major product of 700 bp and four minor products of between 200 bp and 3 kbp) and from *Y. enterocolitica* (a major product of 950 bp and four minor products of between 500 bp and 1.5 kbp) with primers 25–26 (data not shown). The 546 bp *pag* fragment amplified from *B. anthracis* and the above non-specific major fragments were labeled and tested for hybridization with genomic DNA (1 μg) from *B. anthracis*, *Y. pestis* and *Y. enterocolitica*. Therefore, the nucleotide sequences of these PCR products are non-homologous. Each probe only hybridized with the DNA from which it was amplified (data not shown). We hypothesize that the DNA of *Y. pestis* carries sequences of partial homology allowing hybridization of primers 25 and/or 26.

3.2. Multiplex PCR assays

In preliminary experiments using mixtures of primer pairs neither the number nor the size of DNA fragments was modified (unpublished data). We tested whether simultaneous amplification of *lef*, *cya*, *pag* and *cap* was feasible. Primer sets from Table 2 were pooled so that all of the four targeted genes would be amplified once per assay (16 combinations tested consisting of 8 sets). DNA fragments with the expected size were successfully amplified using nearly all combinations (Fig. 1). However, a minor amplified product of about 500 bp is present in all experiments involving the primer pair 3/4. To this point we noted that: (i) single amplification using only the primer pair 3/4 did not generate this fragment, and neither did single amplifications using primer 3 or 4 separately; (ii) the amplification of the 385 bp DNA fragment (3/4) is significantly hampered in multiplex conditions compared to single condition (data not shown). From that, we hypothesize that an illegitimate primer pair, involving primer 3 or 4 and another one present in the mixture, may occur in multiplex conditions, thus allowing a slight amplification of the 500 bp fragment from the genome of *B. anthracis*. The combination of primer pairs 67–68, 25–26, 3–4 and 57–58 primer pairs was the most reliable one in terms of amplification efficiency and gave easily recognizable patterns, thus making DNA fragment profiles easy to interpret for plasmid composi-

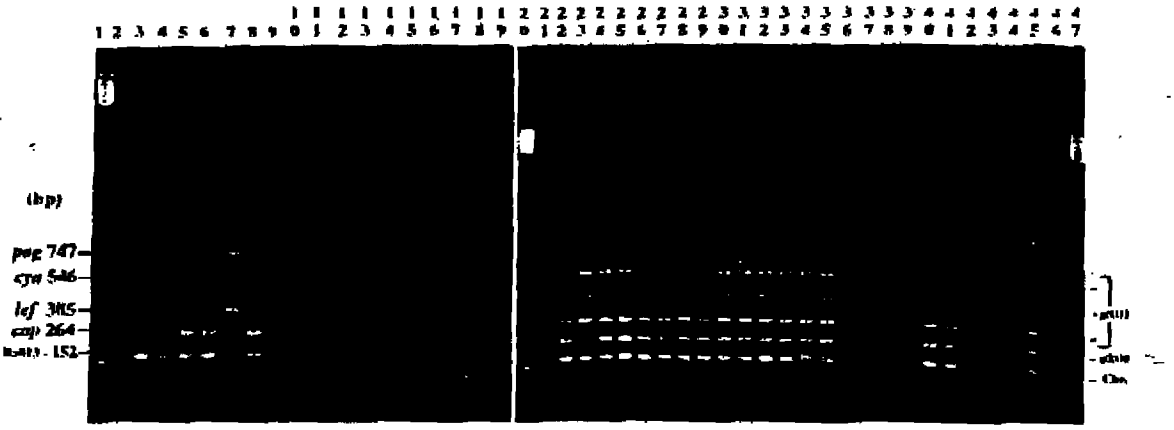

Fig. 2. Results of the amplification of total genomic DNA (~100 ng) by 67/68, 25/26, 57/58, 3/4 and R1/R2 primers. 123 bp ladder (lanes 1,20,47), negative control (lanes 2,21), *B. anthracis* Sterne 7700 (lane 3), Sterne 7702 (lane 4), pXO1$^-$/2$^+$ ATCC4429 (lane 5), pXO1$^-$/2$^+$ ATCC6602 (lane 6), Cepanzo (lane 7), Davis TE702 (lane 8), *B. subtilis* ATCC6051 (lane 9), *B. pumilus* ATCC7061 (lane 10), *B. licheniformis* ATCC14580 (lane 11), *B. stearothermophilus* ATCC7953 (lane 12), *B. thuringiensis* ATCC10792 (lane 13), *B. sphaericus* ATCC14577 (lane 14), *B. alvei* ATCC6344 (lane 15), *B. megaterium* ATCC14581 (lane 16), *B. pasteurii* ATCC11859 (lane 17), *B. cereus* ATCC14579 (lane 18), *B. subtilis* ATCC9372 (lane 19), *B. anthracis* 227 (lane 22), 957 (lane 23), 170 (lane 24), 300 (lane 25), 779 (lane 26), 832 (lane 27), 663 (lane 28), 376 (lane 29), 846 (lane 30), 256 (lane 31), 582 (lane 32), 282 (lane 33), 85 (lane 34), 576 (lane 35), 955 (lane 36), 554 (lane 37), 657 (lane 38), 516 (lane 39), 346 (lane 40), 552 (lane 41), 470 (lane 42), 575 (lane 43), 204 (lane 44), 893 (lane 45) and 69 (lane 46).

tion. Single PCR, targeting virulence factor genes one by one, has been used to characterize *B. anthracis* isolates [9]. Our system, by targeting several virulence factor genes together in the same reaction mixture, makes plasmid content analysis of pure cultures of *B. anthracis* much simpler.

This multiplex PCR assay was not suitable for general identification of *B. anthracis* because a strain lacking both pXO1 and pXO2 would not be identified. Thus, a multiplex PCR using 67–68, 25–26, 3–4, 57–58 (plasmid markers) and R1–R2 primers (chromosomal marker) was tested under the same conditions. Amplification of pXO1 and pXO2 associated virulence factors was still efficient despite the presence of a fifth primer set. The 152 bp DNA fragment from the Ba813 sequence was also efficiently amplified. The specificity of the PCR assay using these five primer pairs was tested against 31 *B. anthracis* isolates and 12 *Bacillus* strains from 11 other species (Fig. 2). The 152-bp DNA fragment was successfully amplified from each of the 31 strains of *B. anthracis* tested but not from other *Bacillus* species, thus confirming species identity. No extra PCR products were generated from closely related members of the *B. cereus* group (Fig. 2). Six well known avirulent strains and 25 isolates of *B. anthracis* were then successfully screened for plasmid content. Each possible type of *B. anthracis* was correctly identified (pXO1$^+$/2$^+$, pXO1$^+$/2$^-$, pXO1$^-$/2$^+$, pXO1$^-$/2$^-$). Three isolates of *B. anthracis* were characterized as pXO1$^+$/2$^-$ (CEB 957, 575 and 69), one as pXO1$^-$/2$^-$ (CEB 554). The others were characterized as fully virulent (pXO1$^+$/2$^+$). Specialized laboratories involved in anthrax control and research use elaborate techniques for confirming strain identity: toxin antigen detection [9], detection of pXO1 and pXO2 virulence factor genes by hybridization with DNA and oligonucleotide probes [7,9,20] or by PCR [8,9]. No single existing method is able to screen for plasmid-associated virulence factor genes and simultaneously to differentiate *B. anthracis* (whether or not containing plasmids) from other *Bacillus* species. Our multiplex PCR assay may meet these requirements and is rapid: less than 3 h after picking an isolated colony from an agar plate.

In summary, the method described here is the first

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example of a rapid procedure enabling identification and genotyping of *B. anthracis*, and simultaneous differentiation from other *B. cereus* group bacteria. Identification of *B. anthracis*, independent of the plasmid content is practicable using the chromosomal Ba813 sequence reported by Patra et al. [17] and the outstanding characterization of the whole marker on the both sides of Ba813 sequence will provide more detailed information. *B. anthracis* isolates that carry neither pXO1 nor pXO2 are clearly distinguished from other *Bacillus* species thus enabling plasmid-free isolates and vaccine seed cultures of *B. anthracis* to be followed. Our method also reduces the identification time from the standard 3–6 days to less than 2 days. This powerful methodology will contribute to reducing the risk of a laboratory-acquired infection and should be of great help as a routine test for confirmation of *B. anthracis* in clinical and environmental samples.

Acknowledgments

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Abstract

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